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Defect in Rearrangement of the Most 5′ TCR–Jα Following Targeted Deletion of T Early α (TEA): Implications for TCR α Locus Accessibility

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Summary

To address the role of the TEA germline transcription, which initiates upstream of the TCR–Jαs, in the regulation of TCR–Jα locus accessibility, we created a mouse in which this region has been removed by homologous recombination. Normal development of Tαβ cells and the expression of other TCR α germline transcripts in TEA$^{-/-}$ mice ruled out an exclusive role for TEA in the overall accessibility of the Jα cluster. However, the rearrangement of the most 5′ Jα (Jα61 to Jα53) was severely impaired, indicating that TEA may control the DNA accessibility of a particular Jα window. Moreover, the relative usage of every Jα segment was affected. These results are consistent with TEA acting as a "rearrangement-focusing" element, targeting the primary waves of Vα–Jα recombination to the most 5′ Jαs in an ongoing TCR–Jα rearrangement model.

Introduction

T and B lymphocytes exert their specialized function of immune surveillance through highly diverse antigen receptors, the T cell receptor (TCR) in the case of T lymphocytes. Like immunoglobulin genes, TCR genes are dispersed along the chromosome into several clusters of variable (V), diversity (D), and joining (J) gene segments, which are somatically rearranged to form functional gene units (for a recent review see Lewis, 1994). The V(D)J recombination process is initiated by the recognition of recombination signal sequences (RSSs), which flank all immunoglobulin and TCR gene segments and are composed of heptamers and nonamers separated by either 12 or 23 bp. The lymphoid-specific recombination-activating gene products RAG1 and RAG2 (Schatz et al., 1989; Oettinger et al., 1990) are necessary and sufficient to initiate V(D)J recombination through the generation of a DNA double-stranded break (McBlane et al., 1995; van Gent et al., 1995). The ubiquitously expressed DNA repair machinery of the cell is then recruited to complete the reaction (for review see Weaver, 1995). V(D)J recombination plays an essential role in generating the necessary immune diversity of B and T cells and is an obligatory step in the maturation program of these cells (Mombaerts et al., 1992b; Shinkai et al., 1992).

Although the V(D)J recombinase is common to both B and T lymphocytes (Yancopoulos et al., 1986), its activity is spatially and temporally regulated; for example, TCR gene rearrangements proceed only in T cells, with TCR β rearrangements preceding recombination at the TCR α locus. The "accessibility model" (Yancopoulos et al., 1986) was proposed to account for the regulation of V(D)J recombination. The model states that only genes that have their chromatin in an accessible (open) configuration serve as substrates for the recombinase. A corollary of this model is that transcriptional activity can be demonstrated surrounding the recombining genes just prior to their actual rearrangement, although it is still unclear whether transcription is itself responsible for gene accessibility or merely reflects the changes in chromatin structure. Although the molecular mechanisms ensuring the regulation of the recombinase are still poorly defined, many studies have reported on the essential role of transcriptional enhancers in V(D)J joining. This has been illustrated first by their obligatory presence within mini-locus recombination substrates in several transgenic models (Bucchini et al., 1987; Goodhardt et al., 1987; Ferrier et al., 1990; Capone et al., 1993; Lauster et al., 1993; Lauzurica and Krangel, 1994; Okada et al., 1994) and more recently through their targeted deletion via homologous recombination (Chen et al., 1993; Serwe and Sablitzky, 1993; Takeda et al., 1993; Bouvier et al., 1996; Bories et al., 1996). In the case of the immunoglobulin κ (Igκ) locus, the 3′ transcriptional enhancer has been found to exert both positive and negative regulatory effects on Vκ–Jκ rearrangements (Hiramatsu et al., 1995).

T lymphocytes are further divided into two subpopulations expressing either the α/β or γ/δ TCR complex (for a recent review see Kisielow and von Boehmer, 1995). The emergence of γ/δ lymphocytes within the developing thymus around day 13 of gestation in the mouse precedes the onset of Tα/β cells (day 16–17) (Pardoll et al., 1987). The question of the lineage relationship between Tγ/δ and Tα/β cells has been controversial, and various models of lineage commitment have been proposed. We and others have recently provided evidence for a late common progenitor for these two lineages through the finding of TCR δ rearrangements in αβ precursors (Dudley et al., 1995; Livak et al., 1995; Wilson et al., 1996). The TCR α/δ locus provides a unique situation, with the Dδ, Jδ, and Cδ gene segments embedded within the TCR α locus between the Vα and Jα elements both in humans and mice (Chien et al., 1987; Hockett et al., 1988). Despite their physical association, the TCR δ and α loci are independently rearranged and expressed during thymic ontogeny. This implies the existence of highly efficient regulatory mechanisms controlling DNA accessibility within the two loci, in particular precluding any Vα–Jα rearrangement in Tγ/δ cells, which would cause the deletion of the TCR δ locus.

We have described the production of germline transcripts, termed TEA (T early α), in human thymocytes

that initiate upstream of the most 5′ Jα element (J61) (de Villartay et al., 1987). The TEA transcripts have subsequently been found in mouse thymi (Hockett et al., 1989; Shimizu et al., 1992). In both species, the TEA promoter is also evolutionarily conserved (de Chasseval and de Villartay, 1993). In addition, we have recently shown that the TEA transcription is confined to the immature single positive cells in the murine thymus, a cell population that is thought to include immediate precursors of Tαβ cells (Wilson et al., 1996). By analogy with what was known about germline transcripts in both immunoglobulin and TCR genes, we have proposed that the TEA transcription could participate in the opening of the Jα cluster to the V(D)J recombinase (de Villartay and Cohen, 1990). To test this hypothesis directly, we generated mutant mice in which the TEA promoter and exon were deleted following homologous recombination in embryonic stem (ES) cells. The analysis of T cell development in homozygous ($TEA^{-/-}$) mutant mice establishes that a TEA regulatory element or TEA transcripts (or both) are not absolutely required for opening the Jα cluster to the V(D)J recombinase, but may control rearrangements of a small group of 5′ Jα segments.

Results

Generation of $TEA^{-/-}$ Mice

A TEA-containing genomic clone was isolated from a 129/Ola genomic library to ensure isogeny between the targeting sequences and the DNA from the ES clone E14.1 (Kühn et al., 1991). Our mutation strategy was to delete 2.3 kb of DNA sequence extending from the TEA promoter (de Chasseval and de Villartay, 1993) down to the Jα61 segment, leaving intact its RSS. In numbering the various Jαs, we refer to the nomenclature of Koop et al. (1994). In this nomenclature, Jα61 (ψJα) represents the first Jα, previously noted as Jα50 (Wilson et al., 1992). The targeting construct (Figure 1A) contains 3.8 kb of DNA sequences upstream of the TEA promoter linked to a *loxP*-flanked *Neo* cassette (Gu et al., 1993) and 7.1 kb of sequences downstream of Jα61. The targeting construct was linearized with BamHI and electroporated into E14.1 ES cells. Two probes (Figure 1A), localized 5′ (probe A) or 3′ (probe RV-1) to the region of homology present in the targeting construct, were used to analyze recombination events by Southern blot. Substitution of the TEA region by the *Neo* cassette leads to a reduced size (10.1 to 9.0 kb) of a KpnI restriction fragment upon hybridization with the probe A (Figure 1B). Out of 391 Neo^r clones, 15 were found to result from a homologous recombination event (3.8%) using the probe A (data not shown). Further Southern blot analysis of one clone (6E1) confirmed that it carried the intended homologous recombination event. Thus, hybridization with probe A yielded a KpnI restriction fragment of a reduced size (from 10.1 to 9.0 kb) owing to the replacement of the TEA region by the Neo^r cassette (Figure 1B). Moreover, a new XhoI site was created, leading to an 8.0 kb fragment upon EcoRV–XhoI digestion and hybridization with the RV-1 probe (Figure 1B).

The inclusion of a selectable marker in the targeting construct has been shown to influence the phenotype of the introduced mutation in the analysis of β-globin-associated regulatory sequences (Fiering et al., 1995). We therefore used the Cre recombinase from phage P1 to eliminate the *loxP*-flanked Neo^r gene from the 6E1 homologous recombinant (Sauer and Henderson, 1988; Gu et al., 1993). A Cre recombinase–expressing plasmid (pIC-Cre; Gu et al., 1993) was transiently transfected into 6E1 cells, and the cells were plated without any selection. Randomly picked clones were then assessed for the loss of the Neo^r gene by polymerase chain reaction (PCR) (data not shown), and one selected clone (6E1C1) was further analyzed by Southern blot (Figure 1B). As predicted, Cre-mediated deletion of the Neo^r gene resulted (Cre allele in Figure 1A) in a 7.8 kb KpnI fragment that hybridized with the A probe, but not with a *Neo* probe. The 8.0 kb EcoRV–XhoI fragment detected with the RV-1 probe remains unaltered on the Cre allele owing to the persistence of the XhoI site as part of the *loxP* site remaining on the chromosome following Cre-mediated deletion.

Clone 6E1C1 was injected into Balb/c blastocysts to generate chimeras. Homozygous ($TEA^{-/-}$) mutant mice were generated by intercrosses of heterozygous ($TEA^{+/-}$) mutants. Offspring were screened by Southern blot using the RV-1 probe and an EcoRV digest (Figure 1C) and showed a normal segregation of homozygous and heterozygous pups.

Normal Development of TCR α/β T Cells in $TEA^{-/-}$ Mice

Thymocyte development is blocked at the $CD4^+CD8^+$ double positive (DP) stage, and mature Tα/β lymphocytes are absent in the periphery in mice carrying a deletion of the TCR α constant region on both alleles (TCR $\alpha^{-/-}$ mice) owing to an absence of TCR αβ expression and subsequent positive selection (Mombaerts et al., 1992a; Philpott et al., 1992). The same situation arises when the early developmental block specific to RAG1/2 knockout mice is overcome by a functional TCR β transgene (Mombaerts et al., 1992a; Shinkai et al., 1993). A similar phenotype would be expected upon deletion of the TEA sequences provided this region is absolutely required for the DNA accessibility of the TCR–Jα cluster to the V(D)J recombinase. Therefore we examined the emergence of TCR αβ–expressing T cells as a first analysis of the effect of the TEA mutation. First, 3 week-old $TEA^{-/-}$ mice have a normal number of thymocytes and peripheral T cells (Figure 2B). Second, normal proportions of mature thymocytes expressing either CD4 or CD8 (presumably TCR $\alpha\beta^+$ thymocytes) develop in $TEA^{-/-}$ mice relative to wild-type controls or $TEA^{+/-}$ heterozygous mice (Figure 2A). Finally, staining of lymph node cells with anti-CD3ε and anti–TCR αβ antibodies (Figure 2A) confirms the existence of TCR αβ–expressing T lymphocytes (about 70%) in this organ at a level similar to that observed in wild-type and heterozygous ($TEA^{+/-}$) mice. A normal proportion of Tγ/δ cells was also found in thymi from day 15 embryonic $TEA^{-/-}$ mice (data not shown), ruling out an effect of the TEA mutation in the development of Tγ/δ cells. Altogether, these results indicate that Vα–Jα rearrangements can occur in the absence of the TEA element and

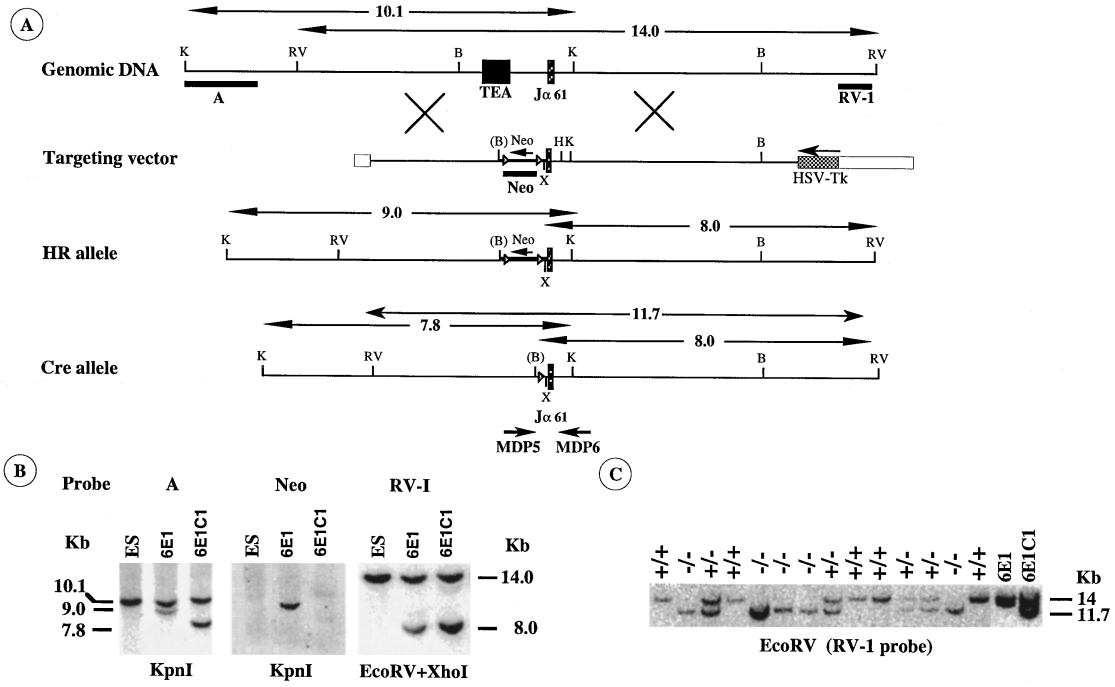

Figure 1. Gene Targeting of the TEA Region in ES Cells

(A) Strategy for homologous recombination. In the targeting vector, 2.3 kb of DNA sequences located immediately upstream of the Jα61 segment and containing the TEA exon and TEA promoter (de Chasseval and de Villartay, 1993) have been replaced by a *loxP*-flanked *Neo* gene. Homologous recombination (HR) results in a reduction to 9.0 kb of the KpnI digestion fragment revealed by the probe A. This fragment is further reduced to 7.8 kb upon Cre-mediated deletion of the *Neo* gene. HR also results in the introduction of an XhoI site in front of Jα61, which leads to the detection of a novel 8.0 kb XhoI–EcoRV fragment with the RV-1 probe. This XhoI site flanks the *loxP* sequences that remain on the chromosome following deletion of the *Neo* gene. The location of the MDP5 and MDP6 primers is indicated on the Cre allele.

(B) Southern blot analysis of HR (6E1) and *Neo*-deleted (6E1C1) ES clones with probes A, *Neo*, and RV-1. Probe A is a 5′ probe, chosen outside of the homologous region, that recognizes the 10.1 kb wild-type, 9.0 kb HR, and 7.8 kb Cre KpnI-specific fragments. The 9.0 kb HR allele is the only fragment hybridizing with the *Neo* probe. The lower hybridization signal of the HR-specific fragment compared with the wild-type allele is probably due to contaminating feeder cells. The absence of hybridization of the 7.8 kb KpnI fragment with the *Neo* probe attests to the excision of the *Neo*^{r} gene and production of the Cre allele. RV-1 is a probe located 3′ of the homologous region that hybridizes to the 14.0 kb wild-type allele and the 8.0 kb HR and Cre alleles following EcoRV–XhoI digestion.

(C) Genotyping of progeny by Southern blot using the RV-1 probe on EcoRV-digested tail DNA. The locations of the 14 kb fragment (wild-type allele) and the 11.7 kb fragment (mutated allele) are indicated.

K, KpnI; RV, EcoRV; B, BglII; H, HpaI; X, XhoI. The triangles on either side of the *Neo* cassette represent the *loxP* sites flanking the *Neo* cassette.

hence that this element is not essential for the opening of the TCR–Jα cluster to the V(D)J recombinase.

TCR α Germline Transcription in TEA$^{-/-}$ Mice

The occurrence of Vα–Jα rearrangements in the absence of the TEA element suggested that other regulatory regions might be responsible for the opening of the TCR–Jα cluster. Since the presence of germline transcripts generally reflects chromatin accessibility, we searched for such transcripts, other than TEA, in the TEA$^{-/-}$ mice. The breeding of RAG2$^{-/-}$ mice into TCR β transgenics (RTB model) and the in vivo treatment of RAG2$^{-/-}$ mice with anti-CD3 (RT3 model) both induce the development of thymocytes up to the DP stage (Mombaerts et al., 1992a; Levelt et al., 1993; Shinkai et al., 1993; Jacobs et al., 1994; Shinkai and Alt, 1994). The thymi obtained from RAG2$^{-/-}$ mice treated with anti-CD3 for 9 days and RTB mice are similar both in terms of cellularity and distribution of the CD4 and CD8 markers, and the TEA transcripts are expressed in the thymi of the two types of mice (Figure 3; data not shown), in agreement with the notion that the TCR–Jα cluster is in an open yet unrearranged configuration in these mice owing to the absence of RAG2 protein. TEA$^{-/-}$ mice were bred onto RAG2$^{-/-}$ mice, and the resulting TEA$^{-/-}$ RAG2$^{-/-}$ mice were treated intravenously with anti-CD3 monoclonal antibody for 9 days (TEA$^{-/-}$ RT3 mice). Northern blot analysis clearly established (Figure 3) that, while TEA-expressing transcripts were absent in thymi from TEA$^{-/-}$ RT3 mice as expected, other Cα-hybridizing germline transcripts were present. The expression of these transcripts, which initiate in different regions of the TCR–Jα cluster (data not shown), indicates that *cis*-acting elements other than TEA, scattered within the Jα segments, may participate in the regulation of DNA accessibility within the TCR–Jα cluster.

Lack of Detectable Rearrangements at the 5′ End of the Jα Cluster in TEA$^{-/-}$ Mice

To test whether the TEA region might have a quantitative or a qualitative influence (or both) on Vα–Jα rearrangements, we studied TCR α recombination events

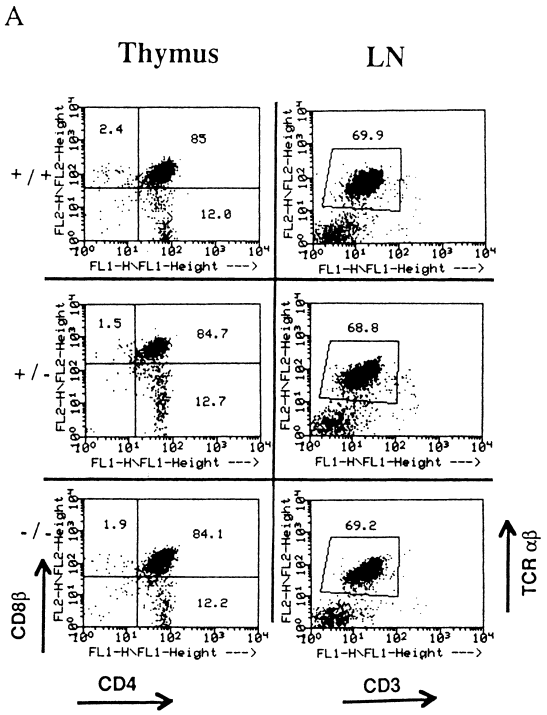

Figure 2. Normal Tαβ Cell Development in TEA$^{-/-}$ Mice

(A) Thymocytes from 3-week-old wild-type, TEA$^{+/-}$ heterozygous, and TEA$^{-/-}$ homozygous littermates were double stained with anti-mouse CD4–FITC and biotin-coupled CD8β (CD8β–BIO) anti-mouse antibodies (revealed by phycoerythrin-coupled streptavidin). Lymph node mononuclear cells from the same mice were labeled with anti–mouse CD3–FITC and anti–mouse pan TCR α/β–BIO.

(B) Number of thymocytes and spleen cells in wild-type and homozygous TEA$^{-/-}$ littermates.

both in peripheral mature Tαβ lymphocytes and in thymocytes from heterozygous and homozygous mutant mice. Highly purified B and Tαβ lymphocytes were obtained by fluorescence-activated cell sorting from spleens and lymph nodes of heterozygous TEA$^{+/-}$ and wild-type mice, and DNA from these cells was used for Southern blot analysis using the RV-1 probe. This probe is located (see Figure 6B) on a 14.0 kb EcoRV germline fragment spanning the TEA region and the most 5′ Jαs (Jα61 to Jα53). The Vα–Jα rearrangements occur by deletion and hence should result in the loss of the 14.0 kb germline hybridization signal. B cells from wild-type mice retain the RV-1 fragment in a germline configuration (Figure 4, lanes 1–3), as expected for an absence of TCR α gene rearrangement in these cells. The same is true for both the wild-type (14.0 kb) and mutated (11.7 kb) alleles in B cells from heterozygous +/− mice (Figure 4, lane 4). In contrast, no RV-1 hybridization signal is retained in mature TCR αβ–expressing T lymphocytes from normal mice (Figure 4, lanes 5–8). The HI-4 probe was used to ensure the integrity of the loaded DNA. This indicates that Vα–Jα rearrangements concerned both alleles in mature Tαβ cells, a result consistent with the absence of allelic exclusion at the TCR α locus (for review see Malissen et al., 1992). Although there is a complete loss of RV-1 hybridization signal corresponding to the wild-type allele in Tαβ cells from heterozygous mice (Figure 4, lanes 9–13), hybridization to the mutated allele (11.7 kb) is retained to a large extent in these cells. This result would imply that the TCR–Jα cluster is in germline configuration on a large proportion of the mutated alleles. Surprisingly, Southern blot analyses of nine Tαβ cell hybridomas generated from heterozygous TEA$^{+/-}$ mice indicated a deletion of the region covered by

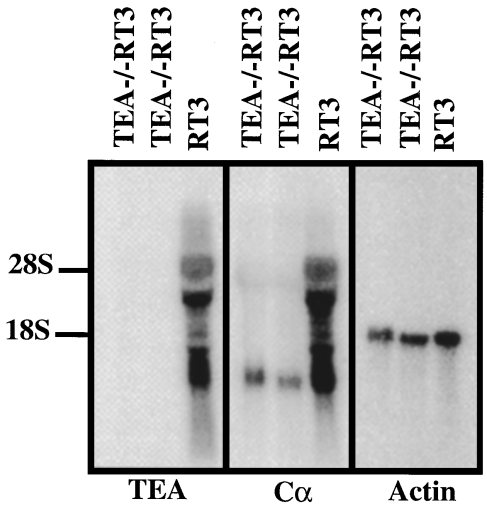

Figure 3. TCR α Germline Transcription in TEA$^{-/-}$ Mice

RAG2$^{-/-}$ and TEA$^{-/-}$RAG2$^{-/-}$ mice were treated intravenously with anti-CD3 (RT3 and TEA$^{-/-}$RT3 mice, respectively), and their thymi were recovered after 9 days. Northern blots of total RNA were sequentially hybridized with a TEA, a Cα, and an actin probe. The positions of the 28S and 18S ribosome are indicated.

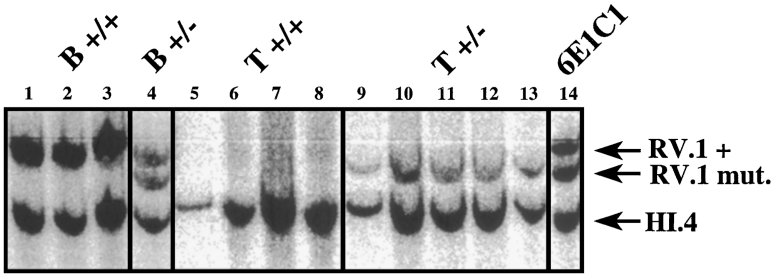

Figure 4. Persistence of Germline 5′ TCR–Jα Sequences in Sorted Mature Tαβ Cells from TEA$^{+/-}$ Mice

Southern blot analysis of DNA from sorted TCR αβ T and B cells from wild-type (T+/+, B+/+) and TEA$^{+/-}$ heterozygous (T+/−, B+/−) mice. DNA was digested with EcoRV, blotted, and hybridized simultaneously with RV-1 and HI-4 probes. The size of the relevant fragment is indicated in kilobases.

the RV-1 probe on both alleles in all of these hybridomas (Figure 5).

These apparently contradictory results were reinterpreted in light of the recent reports by Livak et al. (1995) and Livak and Schatz (1996) showing the persistence of excised DNA circles generated upon Vα–Jα rearrangements in thymocytes and to a lesser extent in peripheral T lymphocytes, but not in T cell hybridomas. According to this interpretation, the persistence of RV-1 hybridization signal on the mutated allele in peripheral T cells from TEA$^{+/-}$ mice, but not in hybridomas obtained from these cells, may reflect the presence of Vα–Jα excised circles rather than bona fide germline sequences. The EcoRV fragment hybridizing with the RV-1 probe carries nine Jα segments (Jα61 to Jα53). Our results therefore suggest that these segments were not involved in Vα–Jα recombination; the Vα–Jα rearrangements originate downstream of the Jα53 on alleles carrying the TEA mutation.

To support the above interpretation, we performed quantitative Southern blot analyses of Jα rearrangements in thymi from mice homozygous for the TEA deletion and wild-type littermate controls (Figure 6). We utilized a series of nine probes (Figure 6B) that span the entire Jα cluster (Rytkonen et al., 1994) as well as sequences upstream of Jα61 and allow for the surveillance of any Jα rearrangement using two restriction digests, EcoRV for the 5′ half and HpaI for the 3′ part of the locus (Rytkonen et al., 1994). The HI-5 probe was used for quantitation purposes. Graph representations of signal quantitation for the entire TCR–Jα locus in TEA$^{-/-}$ versus wild-type mice are shown in Figure 6B; the hybridization signal obtained with the probe A, which is located upstream of the first Jα (Jα61), is retained at 75% (lower graph) and 85% (upper graph) in +/+ and −/− thymus, respectively, consistent with these sequences representing excision products of Vα–Jα rearrangements, as recently documented by Livak et al. (1995) and Livak and Schatz (1996). We noticed a sharp decrease in the intensity of the hybridization signal (17% signal retained) with the downstream probe, RV-1, in the thymus from wild-type mice. This attests to a participation (or a deletion) of the underlying Jα segments in Vα–Jα rearrangements in wild-type thymus. In striking contrast, most of the RV-1 hybridization signal is retained (90%) in the thymus DNA from homozygous TEA$^{-/-}$ mice, indicating that most of the Jα61 to Jα53 segments are present in germline configuration in these mice. Interestingly, the hybridization signal obtained with the next downstream probe, RV-2K, which spans Jα52 to Jα48, is reduced to a similar extent in thymus from both +/+ (22% retained) and −/− (31% retained) mice, indicating that these Jαs are implicated (or deleted) in Vα–Jα rearrangements in both types of mice. This suggests that the majority of the germline Jα61 to Jα53 elements found in TEA$^{-/-}$ mice (RV-1 probe) reside on Vα–Jα excised DNA circles rather than on chromosomal unrearranged alleles. To address this issue more directly, we performed pulsed-field gel electrophoresis using the PacI restriction enzyme. Recognition sites for the PacI enzyme flank the TCR α/δ locus, leading to germline fragments of 95–100 kb upon digestion depending on the strain of mice (Nakajima et al., 1995). The RV-1 probe hybridizes to a germline 100 kb band in the L929 fibroblasts (Figure 6C), whereas no hybridization signal is detected in TEA$^{-/-}$ and wild-type thymi, arguing that no germline TCR α/δ loci are retained in these samples.

Altogether, these results confirm that the retention of the RV-1 signal first noted in TEA$^{+/-}$ peripheral T cells was not due to a massive block of Vα–Jα rearrangement on the mutated allele, but rather reveal that the TEA mutation strongly compromised rearrangements on the first nine Jα segments (Jα61 to Jα53), thus defining a distinctive window of Jα segments, the rearrangements of which are under the influence of the TEA region.

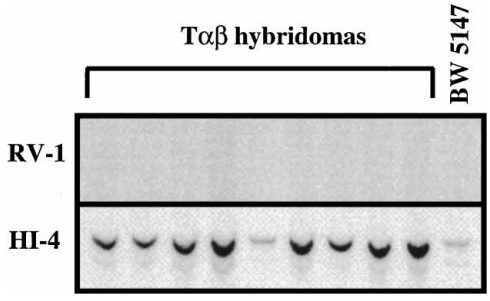

Figure 5. Absence of 5′ TCR–Jα Sequences in Tαβ Cell Hybridomas from TEA$^{+/-}$ Mice

The rearrangement status of the TCR–Jα locus in a series of nine Tαβ hybridomas from TEA$^{+/-}$ mice was assessed by Southern blot analysis using the RV-1 probe on EcoRV restriction digest. Hybridization with the HI-4 probe attests to the integrity of DNA samples.

Modification of Jα Usage in TEA$^{-/-}$ Mice

To define more precisely the effect of TEA deletion on Vα–Jα rearrangements, we analyzed the amount of Jα recombination at the single Jα level by determining the Jα usage in transcripts from peripheral Tαβ cells in mutant mice. TCR α–specific transcripts from lymph node T cells were reverse transcribed and PCR amplified using

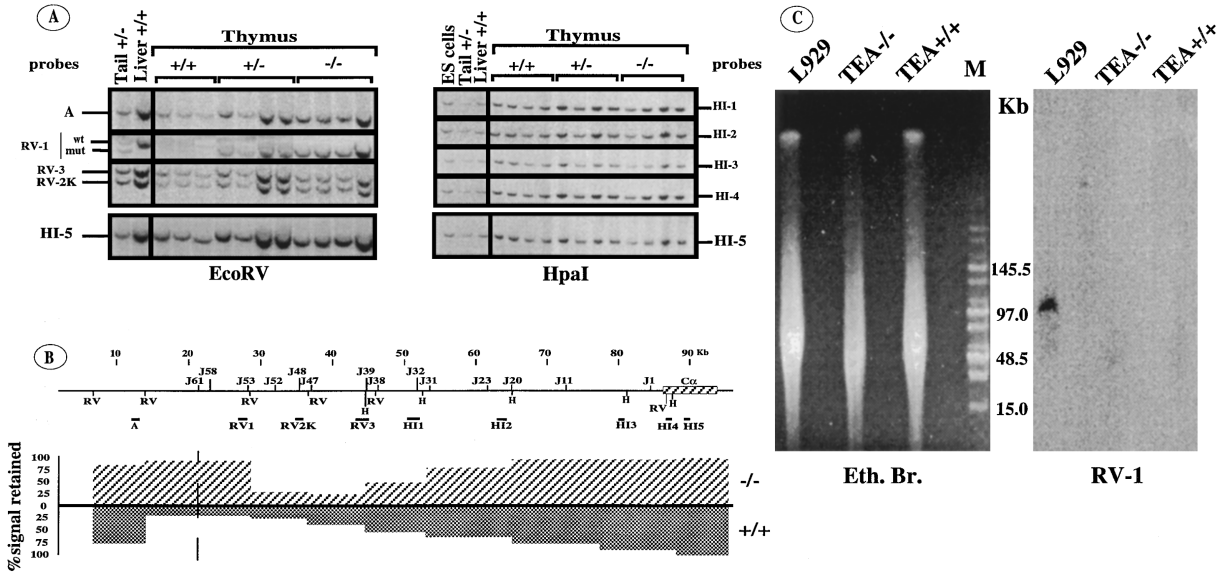

Figure 6. Altered Patterns of TCR–Jα Rearrangements in TEA$^{-/-}$ Thymocytes

(A) Southern blot analysis of DNA from wild-type, TEA$^{+/-}$, and TEA$^{-/-}$ thymocytes. DNA was digested with EcoRV or HpaI, blotted, and hybridized sequentially with the A, RV-1, and RV-2K plus RV-3 probes for the EcoRV digest, and with HI-1 to HI-4 probes for the HpaI digest. Both blots were subsequently hybridized with the HI-5 probe for quantitation. Tail and liver DNA were used as germline controls.

(B) Scaled map of the TCR–Jα locus (Wilson et al., 1992; Koop et al., 1994), location of the various probes used in this study, and graph representation of quantitation of the Southern blots shown in (A). Results are expressed as the percentage of signal retained for each probe, calculated following the formula presented in the Experimental Procedures. Each value represents the mean of three and four thymi for wild-type and TEA$^{-/-}$ mice, respectively. For clarity, several RV sites present between J31 and J1 were not included. RV, EcoRV; H, HpaI.

(C) Pulsed-field gel electrophoresis analysis of the TCR–Jα locus. DNA from TEA$^{-/-}$ and wild-type thymocytes was digested with PacI restriction enzyme, subjected to pulsed-field gel electrophoresis, and analyzed by Southern blot using the RV-1 probe. The L929 fibroblasts are included as TCR α/δ germline control.

primers (Table 1) specific for three different Vα gene segments (VαF3, Vα2C, and Vα5H) (Levin et al., 1993) and a Cα primer (MCA1). Southern blots of the PCR products obtained from ten homozygous TEA$^{-/-}$ and three wild-type TEA$^{+/+}$ mice were then hybridized with a series of Jα-specific oligonucleotide probes and a Cα probe for quantitation purposes. The hybridization patterns were similar for the three different Vα primers; results using the VαF3–Cα combination are presented in Figure 7A. Our PCR conditions were not aimed at determining the exact level of Jα usage (the Jα "repertoire") in each mouse, but rather at determining the relative usage of each specifically tested Jα between the mutant and the wild-type mice by calculating the ratio of hybridization signal obtained in both cases (see Experimental Procedures). A faint hybridization signal (less than 5%) is detected when using Jα58 to Jα53 probes in samples obtained from ten different homozygous TEA$^{-/-}$ mice, in contrast with the strong signal obtained with these probes in transcripts from wild-type controls. The weak hybridization in TEA$^{-/-}$ mice was not due to sequence polymorphism in these Jα segments between the 129/Ola allele and the Balb/c allele, since Vα–Cα PCR products from 129/Ola lymph nodes strongly hybridized. These results confirm the extreme reduction in Jα61 to Jα53 usage in T cells from homozygous mutant mice, as first noted by Southern blot analysis in the thymus (Figure 6B). A second series of probes, corresponding to Jα52 to Jα39, demonstrate a significant hybridization in TEA$^{-/-}$ samples, although to a lesser extent compared with signal obtained with T cells from wild-type mice. Quantification of the hybridization gave ratios of signals in knockout versus wild type ranging from 12% to 84% for these probes. Graph representations of these calculated knockout/wild type ratios of Jα usage, plotted as a function of the relative distance between the various Jαs, indicate a nearly linear progression of these values up to Jα39 (Figure 7B). Finally, all the calculated knockout/wild type ratios are greater than 1 for the last series of Jα probes (Jα34 to Jα2), demonstrating that these Jα segments are overrepresented in the mutant TEA$^{-/-}$ mice relative to the wild-type mice. Interestingly, the graph representations of the knockout/wild type ratio of relative Jα usage obtained with the three different Vα primers are very similar. Moreover, the variations in these ratios are minimal among the different mutant mice for a given Jα probe, which permits the drawing of a general pattern of experimentally determined relative Jα usage in the TEA$^{-/-}$ mice (graph VαF3+2C+5H in Figure 7B).

Discussion

In an attempt to define regulatory DNA sequences involved in TCR–Jα accessibility, we have generated a mutant mouse in which the TEA region has been deleted by homologous recombination. The normal development of TCR α/β–expressing T lymphocytes in the TEA$^{-/-}$ mice argues against a decisive role for TEA in

Table 1. Oligonucleotide Primers and Probes

Primers		
MCA1	5′-ACTGGGGTAGGTGGCGTTGGTCTCT-3′	
MVAF3	5′-ACCCAGACAGAAGGCCTGGTCACT-3′	
MVA2C	5′-ACTGTCTCTGAAGGAGCCTCTCTG-3′	
MVA5H	5′-CAGAAGGTGCAGCAGAGCCCAGAA-3′	
Probes		**Hybridization Temperature (°C)**
MJA58	5′-AGACCCAGTGCCTTGCTGCA-3′	50
MJA57	5′-CGCAGACCCTCCTTGATTCA-3′	50
MJA56	5′-ATTATTGCCTCCAGTAGCCA-3′	45
MJA55	5′-AGGCCATTGTTAGCCTTGC-3′	48
MJA53	5′-GTAATTGCTGCCTCCACTGT-3′	45
MJA52	5′-TCCAGTGTTAGCTCCAGTGT-3′	42
MJA50	5′-GCTGAAGGAGGAGGATGCTA-3′	46
MJA48	5′-TTCTCATTTCCATAGTTGGC-3′	42
MJA45	5′-ATCTGCACCTTCTGTATTCA-3′	40
MJA44	5′-TTTCCACCACTGCCAGTAAC-3′	45
MJA42	5′-TGCATTGCTTCCTCCAGAAT-3′	48
MJA39	5′-GGCACCTGCATTATTATTCA-3′	42
MJA34	5′-TTTGTTGGTATTGGAAGA-3′	37
MJA32	5′-GTTGCCACTGCTCCCATA-3′	45
MJA30	5′-TTGTAAGCATTTGTGTCA-3′	37
MJA28	5′-GTTACTCCCAGTGCCTGGTA-3′	45
MJA22	5′-TTGCCAGCTGCCAGAAGATG-3′	50
MJA17	5′-GTTCCCTGCACTGTTAGTCA-3′	42
MJA12	5′-CTTTATAGCCTCCAGTCCCC-3′	45
MJA5	5′-GCCCCACAACCTGTGTCCCC-3′	55
MJA2	5′-ACCACTTAGTCCTCCAGTAT-3′	37
MCA5	5′-CAAAGTCGGTGAACAGGCAGAG-3′	50

opening the Jα cluster. However, alterations in the pattern of Jα rearrangements in the thymus and Jα usage in the periphery of TEA$^{-/-}$ mice indicate that the TEA region may participate in the control of Vα–Jα gene rearrangement.

TEA and DNA Accessibility in the TCR–Jα Cluster

Many transcriptional *cis*-regulatory sequences are present in the TCR α/δ locus that could participate in the regulation of chromatin accessibility. Both TCR α and TCR δ have their own transcriptional enhancers (Ho et al., 1989; Bories et al., 1990; Redondo et al., 1990), which have been shown to be essential for V(D)J recombination in transgenic experiments. The TCR δ enhancer is active in both α/β and γ/δ cells and plays a key role in rendering the Jδ accessible to the recombinase, while it is dispensable for Vδ–Dδ rearrangements to occur (Lauzurica and Krangel, 1994). Similarly, the TCR α enhancer confers both tissue and stage specificity on V(D)J recombination within TCR α/β–expressing T cells (Capone et al., 1993). Although a decisive characterization their role awaits gene targeting experiments, mutations of this type of element in both the heavy and light chain immunoglobulin loci strongly support this hypothesis (Chen et al., 1993; Serwe and Sablitzky, 1993; Takeda et al., 1993). Moreover, some of us (Bouvier et al., 1996) and others (Bories et al., 1996) have recently demonstrated that the targeted deletion of the TCR β enhancer (Eβ) results in the absence of TCR β gene rearrangement. Other *cis*-acting elements are likely to influence chromatin accessibility in the TCR α/δ locus. A locus control region (LCR) has been described downstream of the TCR α enhancer in transgenic experiments (Diaz et al., 1994). A LCR has first been demonstrated in the β-globin locus, where it is responsible for the ordered expression of the various embryonic, fetal, and adult globin genes (for review see Crossley and Orkin, 1993; Dillon and Grosveld, 1993). The LCR, however, does not influence chromatin accessibility by itself, but rather through its interaction with promoters and enhancers, presumably by protein–protein interactions (Reitman et al., 1993). It has been proposed that the TCR α/δ LCR could regulate V(D)J rearrangements through its competitive interactions with either δ- or α-specific *cis*-regulatory sequences (Diaz et al., 1994). Recently, a novel class of regulatory sequences with both rearrangement "silencing" and "anti-silencing" activities has been described upstream of the Jλ locus in the chicken (Lauster et al., 1993). A similar element was found upstream of the murine Jκs, the targeted mutation of which profoundly, although not totally, impairs Vκ–Jκ rearrangements (Ferradini et al., 1996). These sequences have thus been proposed as "rearrangement-enhancing" elements. Except for the nine most 5′ Jα segments, the TCR–Jα locus is rearranged in the TEA$^{-/-}$ mice, arguing in favor of the existence of several targets for the putative DNA decondensation activity of the Eα, the LCR, or both. Interestingly, the entire 60 kb of DNA sequences spanning the TCR–Jα cluster are evolutionarily conserved between humans and mice (71% homology), with local region similarities up to 90% (Koop and Hood, 1994). However, coding sequences (Jα) in this region account for less than 6%, which argues for the likely strong selective pressure on noncoding, supposedly *cis*-regulatory sequences in this locus. Accordingly, TCR α–specific germline transcripts other than TEA are found in the

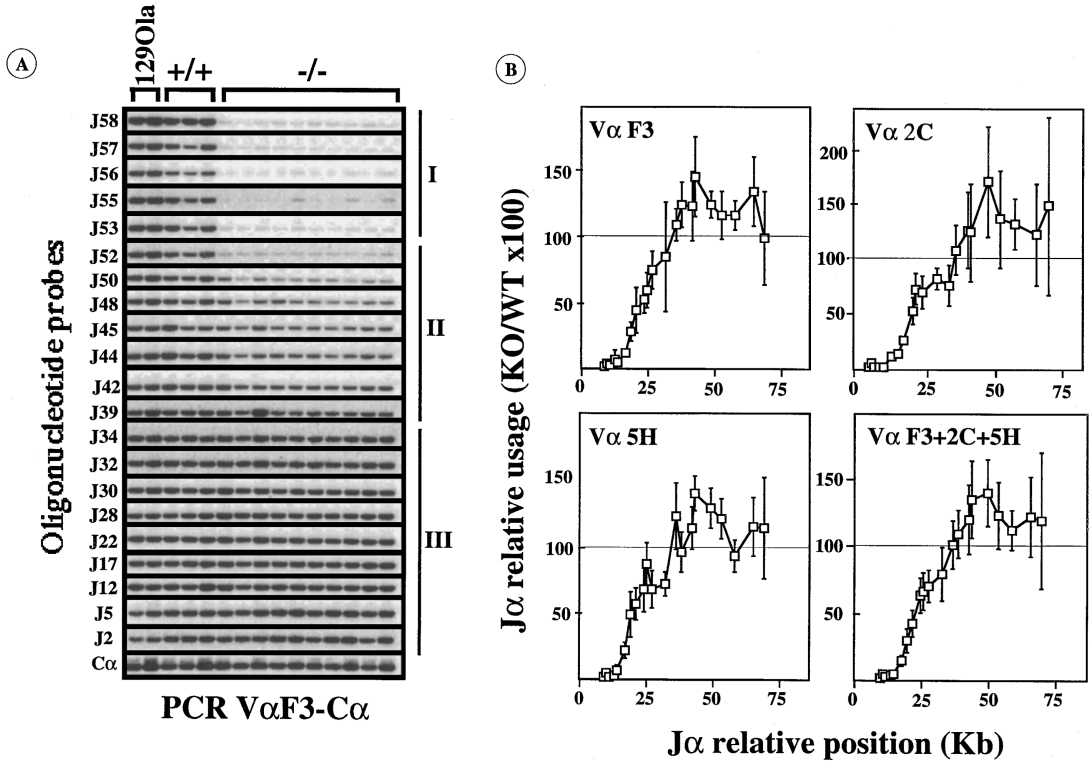

Figure 7. Relative TCR–Jα Usage in Peripheral T Cells from TEA$^{-/-}$ and Wild-Type Mice

(A) Profile of Jα usage in peripheral lymph nodes of ten homozygous TEA$^{-/-}$ mice and three wild-type control littermates. Vα–Cα reverse transcription–PCR was performed with the MVAF3 and MCA1 primers (Table 1), electrophoresed on 1% agarose gel, blotted onto nylon membrane, and hybridized with the indicated Jα-specific oligonucleotide probes as well as the MCA5 Cα-specific probe (Table 1).

(B) Graphic representation of the Jα knockout/Jα wild type ratio obtained following quantitation of the PCR products for VαF3 (shown in [A]) and for Vα2C and Vα5H (data not shown). Each Jα is positioned on the X axis according to its real position on the TCR–Jα cluster sequence. The lower right graph represents a compilation of the values obtained for VαF3, Vα2C, and Vα5H PCRs.

TEA$^{-/-}$ mice, the promoters of which could indeed participate to the overall accessibility of the TCR–Jα locus (Figure 3). The TEA mutation consisted of a 2.3 kb deletion. Further analyses are now required to define more precisely the core region responsible for the observed phenotype. However, no obvious *cis*-regulatory sequences, such as enhancers, other than the TEA promoter could be found in the human equivalent region by transient transfection assays (J.-P. d. V., unpublished data). The TEA mutation results in a profound decrease of rearrangement on the first Jα sequences (Jα61 to Jα53). This suggests that the DNA sequence covering Jα61 to Jα53 segments represents an independent window of rearrangement under the control of TEA. Whether the totality of the Jα cluster can be subdivided into several such windows remains an open question that needs to be considered in light of two other specific attributes of the TCR α locus: first, the possibility of ongoing Vα–Jα rearrangements and, second, the relative Vα–Jα rearrangement synchronism on both alleles.

Ongoing Vα–Jα Rearrangements

The TCR α locus presents an unusual property when compared with other TCR or immunoglobulin genes: the lack of allelic exclusion at the gene rearrangement level (Borgulya et al., 1992; Malissen et al., 1992). This means that both TCR α alleles are generally rearranged in T lymphocytes, leading sometimes to the expression of dual TCR at the cell surface (Malissen et al., 1988; Padovan et al., 1993; Hardardottir et al., 1995). In this context, several reports have provided evidence for the likelihood of multiple Vα–Jα rearrangements on each allele. First, the expression of the *RAG1* and *RAG2* genes is downregulated upon signaling through the TCR in developing thymocytes (Turka et al., 1991), leaving the theoretical possibility of secondary TCR α rearrangements in the absence of appropriate TCR triggering. Second, ongoing Vα–Jα rearrangements have been demonstrated in vitro in a virally transformed immature T cell line (Marolleau et al., 1988; Fondell and Marcu, 1992). Finally, CD3/TCR^{lo} DP thymocytes from bcl2 transgenic mice demonstrated a slight 3′ shift in their TCR–Jα rearrangement pattern when cultured in a nonselective environment, arguing for the conceivable occurrence of secondary Vα–Jα rearrangements during this period in vitro (Petrie et al., 1993, 1995). To test whether the phenotype of the TEA$^{-/-}$ mice agreed with the ongoing Vα–Jα model of rearrangements, we performed a computer-assisted theoretical simulation of Jα usage in the mutant mice (see Experimental Procedures), which took into account successive Vα–Jα rearrangements and resulted in a theoretical curve that superimposed almost perfectly with the experimental values presented in Figure 7 (data not shown).

TEA as a Rearrangement-Focusing Element?

Vα–Jα rearrangements have been described not only as occurring on both chromosomes, but also as involving neighboring Jαs (Malissen et al., 1992; Rytkonen et al., 1994). Such Jα synchronism is difficult to envision in a situation in which the 61 Jα segments have the same probability of being rearranged first, so we favor the hypothesis of a preferential entry at the 5′ side of the Jα cluster. Moreover, this scenario is more readily supported by previous reports in the literature. First, Southern blot analyses of thymic DNA (Figure 6B; Livak et al., 1995) indicate that the level of Jα rearrangements is the strongest at the 5′ side of the Jα cluster and declines progressively thereafter. Second, the analysis of T cell hybridomas obtained from fetal, early postnatal, or adult thymi clearly demonstrated a skewing toward the most 5′ part of the Jα locus in early α/β thymocytes (Thompson et al., 1990). Finally, Livak and Schatz (1996) reported a high level of DNA double-stranded breaks in thymus DNA in the vicinity of Jα61 (Jα50 in their nomenclature). Since DNA double-stranded breaks at RSSs represent the initial event of the V(D)J recombination (McBlane et al., 1995), this result would imply that Jα61 is more susceptible to rearrangements than other Jα segments.

Conclusion

The DNA accessibility is the mechanism invoked for the regulation of V(D)J recombination and is materialized by germline transcription prior to gene rearrangement. The TEA germline transcript (or, perhaps, transcription) was therefore thought to participate in the regulation of TCR–Jα accessibility. The deletion of the TEA exon and TEA-associated promoter clearly establishes that these elements are not solely responsible for the opening of the Jα cluster to the V(D)J recombinase. However, we would like to propose that TEA possesses a "rearrangement-focusing" activity to drive the first wave of Vα–Jα rearrangements at the 5′ end of the locus in a model of multiple Vα–Jα rearrangements. Moreover, the targeting of the first wave of rearrangements on a narrow 5′ Jα window would provide a plausible explanation for the synchronism in Jα recombination. The TEA$^{-/-}$ murine model should help clarify the molecular mechanism involved in maintaining this synchronism.

Experimental Procedures

Production of TEA$^{-/-}$ and TEA$^{-/-}$ RT3 Mice

A TEA-containing genomic clone was isolated from a 129/Ola phage genomic library (the gift of A. Begue). A 3.8 kb BamHI–BglII fragment upstream of the TEA promoter was inserted in front of the *loxP*-flanked *Neo* gene in pLZ-neo (Gu et al., 1993). A 400 bp PCR fragment (XbaI–HpaI) including the most 5′ Jα (Jα61) and its RSS was inserted downstream of the *Neo* cassette. A 7.1 kb 3′ arm (HpaI–SalI fragment) was then inserted downstream of Jα61 at the HpaI site. Finally, a 1.8 kb HSV–TK cassette from pIC19R/MCI-TK (Mansour et al., 1988) was inserted downstream of the 3′ arm, leading to the final targeting construct (Figure 1A). The PCR-amplified RSS Jα61 region was verified by DNA sequencing. This targeting strategy resulted in a 2.3 kb deletion spanning the TEA promoter and the TEA exon. The E14.1 ES cell line was transfected by electroporation (250 V, 600 μF) using 15 μg of BamHI-linearized targeting vector. After a 10 day selection in the presence of G418 and ganciclovir, single colonies were harvested in 96-well microtiter plates. The plates were duplicated 3 days later. The master plates were frozen, and the duplicate plates were screened for homologous recombination by Southern blot analysis (Ramirez-Solis et al., 1992) using KpnI digest and the A probe (Figure 1B). A 3′ probe (RV-1) and a *Neo* probe were used for further analysis of the selected targeted clone 6E1 (Figure 1B). The *Neo* cassette was removed in the 6E1 primary homologous recombinant clone by means of Cre-mediated recombination (Sauer and Henderson, 1988; Gu et al., 1993). 6E1 cells (5×10^6) were transfected with 10 μg of the Cre-expressing plasmid pIC-Cre (Gu et al., 1993). Living cells were trypsinized after 24 hr and reseeded at 1×10^3 cells per 10 mm^2 petri dish without selection. Random colonies were picked 5 days later and screened for the loss of the *Neo* gene by PCR using the deletion-flanking primers MDP5 (5′-CCTTTATCCTTCTGTCTGAG-3′) and MDP6 (5′-GTCCAC TACTGTTCGTTCAC-3′). One clone, 6E1C1, was selected for further analysis by Southern blot (Figure 1B) and for Balb/c blastocyst injection. Chimeras were scored by coat color and mated to Balb/c mice for germline transmission. Genotyping of progeny for the presence of the TEA mutation was performed by Southern blot analysis (Figure 1C).

TEA$^{-/-}$ RT3 mice were obtained by breeding TEA$^{-/-}$ mice into RAG2$^{-/-}$ mice (Shinkai et al., 1992). Double homozygous TEA$^{-/-}$RAG2$^{-/-}$ mutants were then injected intravenously with 50 μg of anti-CD3 monoclonal antibody (MAb) (clone 2C11), and thymi were collected 9 days later for Northern blot analysis.

Cell Preparations, Fluorescence Analysis, and Cell Sorting

Single cell suspensions of lymphoid organs from knockout mice and wild-type littermate controls were prepared on a tissue metal screen. Immunofluorescence analysis were performed with the following fluorescein isothiocyanate (FITC)-conjugated MAbs: anti–mouse CD3 (clone 2C11; Pharmingen, San Diego, CA), anti–mouse CD4 (clone GK1.5; ATCC, Paris, France) and anti–mouse IgM (Fab′$_2$ fragment; Jackson Laboratories, Bar Harbor, ME), the biotin-conjugated anti–mouse pan TCR αβ (clone H57-597; Pharmingen), and anti–mouse CD8β (clone H35; ATCC). Biotin-conjugated MAbs were revealed by incubation with a phycoerythrin-coupled streptavidin (Caltag Laboratories, San Francisco, CA). The anti–mouse CD16 MAb (clone 2-4G2; gift from J. Unkeless) was used for blocking FcRγII and FcRγIII. For purification of B cells and Tαβ cells by fluorescence-activating cell sorting, lymphocytes from spleen and lymph nodes were isolated on a Ficoll gradient (NycoPrep 1.077 animal; NYCOMED PHARMA AS, Oslo, Norway); about 30×10^6 mononuclear cells were first incubated with the FcRγII blocking reagent 2-4G2, followed by staining with anti–mouse IgM and anti–mouse pan TCR αβ MAbs. Purity of sorted B and Tαβ cells was determined by reanalysis on a Facscan flowcytometer and found to be greater than 98%. Tαβ hybridomas from TEA$^{+/-}$ mice were obtained by fusion of concanavalin A–activated lymph node cells with the BW5147 $\alpha^-\beta^-$ thymoma fusion partner (Letourneur and Malissen, 1989).

DNA Probes

The DNA probes covering the entire TCR–Jα cluster (Figure 6B) were derived from the cosmids 23.1 W7, 55W7, and 20.1W7 (the gift of M. Malissen; Malissen et al., 1988). Probe A is a 2.0 kb SspI–PvuI fragment contained within a 10.1 kb KpnI germline fragment. The 950 bp SacI–BglII (RV-1), the 600 bp PstI–KpnI (RV-2K), the 2.3 kb KpnI–HpaI (RV-3), the 2.2 kb EcoRV–HpaI (HI-1), the 1.3 kb EcoRV–HpaI (HI-2), the 986 bp PCR (HI-3), and the 1.3 kb EcoRV–HpaI (HI-4) probes have been described previously (Rytkonen et al., 1994). The HI-5 probe, which covers the Cα exon 4, was PCR amplified from genomic DNA (MCA11 primer, 5′-ACAATCCTTTCAGCGACCTT-3′, and MCA7 primer, 5′-CCAACCAGACCCCAGACAGC-3′). HI-5 hybridizes to 4.5 kb HpaI and 8.8 kb EcoRV fragments. For Northern blot analyses, TEA exon–specific (Shimizu et al., 1992) and Cα-specific probes were used.

DNA Preparation and Southern Blot Analyses

High molecular mass DNA was prepared by proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation (Maniatis et al., 1982). DNA (10 μg) was digested with 4 U/μg of the indicated restriction enzymes, electrophoresed through 0.7% agarose gel,

and transferred onto a Gene Screen Plus nylon membrane (New England Nuclear, Boston, MA). Gel-purified DNA fragments were labeled using [α-^{32}P]dCTP and random hexamers (Feinberg and Volgestein, 1983). Hybridizations were performed at 42°C in 50% formamide–containing hybridization buffer (Maniatis et al., 1982). Autoradiography on photostimulable screens and quantitative analysis were performed on a PhosphorImager with ImageQuant 3.0 software (Molecular Dynamics, Sunnyvale, CA). The following formulae were used to calculate the amount of hybridization signal retained:

percent signal retained = (signal for specific probe in sample/signal for HI-5 probe in sample) × (signal for HI-5 probe in liver or ES cells/signal for specific probe in liver or ES cells) × 100.

The signal for HI-5 was divided by 2 in the calculation for each allele when considering the RV-1 probe, which discriminates between mutant and wild-type alleles.

Pulsed-Field Gel Electrophoresis

Cells were embedded in 0.5% agarose plugs (Incert, FMC BioProducts, Rockland, ME) in 1× PBS, 10 mM EDTA. Plugs containing 1×10^6 cells were treated at 50°C for 24 hr in 1% Sarcosyl, 0.5 mM EDTA, 2 mg/ml proteinase K and dialyzed against Tris–EDTA (18 hr) before treatment with 1 mM PMSF in Tris–EDTA (3 hr at 20°C). Following another 18 hr dialyzation against Tris–EDTA, the plugs were equilibrated in restriction digest buffer, digested overnight with 300 U of PacI, and treated with 0.1 mg/ml RNase (2 hr at 37°C). The plugs were then included in a 1% SeaKem GTG agarose gel (FMC BioProducts) and subjected to pulsed-field gel electrophoresis (6 V/cm, 5–20 s switch ramp, 19 hr at 14°C with 0.5× TBE) using a CHEF-DR II apparatus (Bio-Rad, Richmond, CA). The gel was treated for 30 min with 0.25 N HCl and transferred onto nylon membrane (Gene Screen Plus) in 0.4 N NaOH, 0.6 M NaCl. Hybridization conditions were as above.

RNA Preparation, Northern Blot, and Reverse Transcription–PCR Analyses

Total RNA was prepared from peripheral lymph nodes and thymus using the GUSCN/$CsCl_2$ ultracentrifugation method (Maniatis et al., 1982). Northern blot analyses were carried out using 10 μg of total RNA by conventional methods (Maniatis et al., 1982). cDNA was synthesized by oligo(dT)-primed reverse transcription according to manufacturer recommendations (Superscript II, Life Technologies SARL, Cergy Pontoise, France). Amplifications of TCR α transcripts were performed by PCR with primers described in Table 1. In brief, amplifications were performed with 0.5 U of Taq DNA polymerase (ATCC) for 35 cycles with 62°C annealing in 50 μl of 1× PCR buffer containing 1.5 mM $MgCl_2$. We used 25 pmol of MCA1 reverse primer (antisense Cα) in combination with 25 pmol of MVAF3, MVA2C, or MVA5H forward primers (sense Vα) (Levin et al., 1993). PCR products were electrophoresed on 1% agarose gel and transferred onto a Gene Screen Plus nylon as above. Hybridizations were carried out with γ-^{32}P-end-labeled Jα-specific oligonucleotide probes (Table 1) as described previously (Rieux-Laucat et al., 1993) at the temperatures indicated in Table 1. Autoradiography and quantitation were performed as above. The relative Jα usage in TEA$^{-/-}$ mice compared with wild-type mice was calculated for each Jα probe according to the following formula:

percent Jα usage = (Jα signal from TEA$^{-/-}$ mouse/MCA5 signal from TEA$^{-/-}$ mouse) × (MCA5 signal from wild-type mouse/Jα signal from wild-type mouse) × 100,

where MCA5 corresponds to the Cα-specific oligonucleotide probe.

Theoretical Simulation of Relative Jα Usage in TEA$^{-/-}$ Mice

Let us first consider that q_{wt} represents the probability of rearrangement on a given Jα on the wild-type allele. Let us also consider that each Vα–Jα rearrangement may be followed by a second recombination event, the probability of which is $(1 - p')$. p', which corresponds to the probability of having a productive Vα–Jα rearrangement (0.33) being positively selected, will be a value between 0 and 0.33. For simplicity, we will consider p' equivalent for each Jα segment. The probability of using the $J\alpha_i$ segment can be defined as:

$$P(J\alpha_i)_{wt} = \frac{q_{wt}p' + [P(J\alpha_{i-1})_{wt}](1 - p')}{\Sigma\, P(J\alpha_i)_{wt}}.$$

The same equation can be applied in the case of the mutated allele, where q_{ko} represents the probability of rearranging on a particular Jα segment considering that the first nine Jα segments are blocked. This formula was computerized with Excel-4 software, the ratio $P(J\alpha_i)_{ko}/P(J\alpha_i)_{wt}$ was calculated for each Jα, and the values thus obtained were plotted against the Jα relative positions.

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